Genome-wide RNAi Screening Identifies RFC4 as a Factor That Mediates Radioresistance in Colorectal Cancer by Facilitating Nonhomologous End Joining Repair

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Abstract

**Purpose**: Neoadjuvant chemoradiotherapy (neoCRT) is a standard treatment for locally advanced rectal cancer (LARC); however, resistance to chemoradiotherapy is one of the main obstacles to improving treatment outcomes. The goal of this study was to identify factors involved in the radioresistance of colorectal cancer and to clarify the underlying mechanisms.

**Experimental Design**: A genome-wide RNAi screen was used to search for candidate radioresistance genes. After RFC4 knockdown or overexpression, colorectal cancer cells exposed to X-rays both in vitro and in a mouse model were assayed for DNA damage, cytotoxicity, and apoptosis. Moreover, the regulatory effects and mechanisms of RFC4 in DNA repair were investigated in vitro. Finally, the relationships between RFC4 expression and clinical parameters and outcomes were investigated in 145 patients with LARC receiving neoCRT.

**Results**: RFC4, NCAPH, SYN3, LDLRAD2, NHP2, and FICD were identified as potential candidate radioresistance genes. RFC4 protected colorectal cancer cells from X-ray-induced DNA damage and apoptosis in vitro and in vivo. Mechanistically, RFC4 promoted nonhomologous end joining (NHEJ)-mediated DNA repair by interacting with Ku70/Ku80 but did not affect homologous recombination-mediated repair. Higher RFC4 expression in cancer tissue was associated with weaker tumor regression and poorer prognosis in patients with LARC treated with neoCRT, which likely resulted from the effect of RFC4 on radioresistance, not chemoresistance.

**Conclusions**: RFC4 was identified as a radioresistance factor that promotes NHEJ-mediated DNA repair in colorectal cancer cells. In addition, the expression level of RFC4 predicted radiotherapy responsiveness and the outcome of neoadjuvant radiotherapy in patients with LARC.

Introduction

Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer mortality worldwide, accounting for more than 9% of all cancer-related deaths (1).

Although the incidence of colorectal cancer has declined in developed countries in recent years, it has been dramatically rising in China (1–3). Neoadjuvant radiotherapy/chemoradiotherapy (neoRT/CRT) is advocated by National Comprehensive Cancer Network (NCCN) guidelines as the standard treatment for locally advanced rectal cancer (LARC) because it has been found to significantly increase local control and cancer-specific survival in these patients (4–6). Most patients with LARC treated with neoRT/CRT exhibit variable degrees of a tumor response, but resistance still occurs in a proportion of patients. To classify these patients, the neoadjuvant rectal (NAR) score, which incorporates weighted cT, ypT, and ypN, has been proposed (7). However, application of the NAR score may be limited by its inability to explain why some patients classified within the same NAR category demonstrate different treatment responses (7, 8). An increasing number of investigations have revealed that the treatment response of individual patients with colorectal cancer is associated with the genetic characteristics and gene expression pattern of cancer cells (9) and that the combined use of molecular biomarkers and clinical parameters would improve the prediction of neoadjuvant therapeutic responses and prognosis for patients with colorectal cancer (10, 11). Nevertheless, few biomarkers have been clinically validated as predictive of the radiotherapy response. Thus, there is an urgent need to elucidate the mechanisms underlying radiotherapy resistance and
Wang et al.

Translational Relevance
Neoadjuvant radiotherapy (neoRT) significantly increases the resection rate and local control of locally advanced rectal cancer (LARC) and the cancer-specific survival of affected patients. Although most patients with LARC treated with neoRT exhibit variable degrees of a tumor response, a proportion of patients still suffer from resistance. Therefore, it is necessary to elucidate the underlying mechanisms of radioresistance and to discover reliable biomarkers for predicting radiosensitivity in patients with LARC. This study identified RFC4 as a radioresistance factor that promotes nonhomologous end joining (NHEJ)-mediated DNA repair in colorectal cancer. RFC4 upregulation in cancer cells was found to be associated with reduced tumor regression and poor prognosis in patients with LARC treated with neoRT. Therefore, a high RFC4 expression level may suggest radioresistance, and RFC4 silencing/inhibition is a potential strategy for radiosensitization in these patients; these findings may contribute to the development of precise treatment strategies in clinical practice in the future.

Materials and Methods
Cells, plasmids, siRNAs, and transfections
The HCT116, HT29, HCT15, and SW480 colorectal cancer cell lines, the NCM460 normal human colon mucosal epithelial cell line, and 293T cells were purchased from ATCC and cultured according to ATCC guidelines. All cell lines were authenticated by short tandem repeat analysis at China Center for Type Culture Collection, and the absence of contamination was verified and inserted into pcDNA4.0 for expression with a Myc or Flag tag. Negative control (NC) and specific siRNAs (Supplementary Table S1) were synthesized by GenePharma. All transient transfections were performed with Lipofectamine 2000 or 3000 (Invitrogen) according to the manufacturer’s guidelines.

Generation of stable cell lines
Human RFC4 cDNA was cloned into the pCDH-EF1-MCS-T2A-Puro plasmid (System Biosciences) tagged with 3 × Flag at the N-terminus; shRNAs (Supplementary Table S2) were cloned into the pLKO.1 vector (Sigma-Aldrich), according to the manufacturer’s guidelines. These plasmids were cotransfected into 293T cells with a Lentiviral Packaging Kit (FulenGen) to obtain recombinant lentiviruses. Colorectal cancer cells with stable RFC4 overexpression or knockdown were obtained by lentiviral transduction and puromycin selection, as described previously (14, 15).

High-throughput RNA interference screening
Screening was performed using the LentiPlex human whole-genome shRNA library targeting 15,000 human genes (an average of 5 shRNAs per target gene; SHPH01; Sigma-Aldrich) based on the manufacturer’s instructions. The relative copy number of each shRNA in the X-ray–treated and untreated groups was compared, and MAGeCK-negative selection was performed to identify candidate radioresistance genes (16, 17). As the knockdown of radioresistance genes confers sensitivity to radiation, cells containing radioresistance gene–targeted shRNAs would be more susceptible to death upon X-ray exposure, and the copy number of these shRNAs would be reduced in the residual cell populations after X-ray treatment. Thus, radioresistance gene candidates should meet the following criteria: (i) log2 (fold change) < −2.0; (ii) FDR (computed from the empirical permutation testing) < 0.05 and FDR rank < 1,000; and (iii) at least three shRNAs with a copy number reduced by more than 50% in X-ray–treated cells compared with untreated cells (Fig. 1A).

Reverse transcription and quantitative PCR
Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using an M-MLV Reverse Transcriptase Kit (Promega). Gene expression levels were assessed using qPCR with iTaq SYBR Green Mix (Bio-Rad) and specific primers (Supplementary Table S3). Relative mRNA levels are presented as 2^(-ΔΔCt) (GAPDH was used as an internal control) after normalization to the control group (18).

Cytotoxicity assays
Cytotoxicity or cell sensitivity to X-rays or drugs was analyzed using colony formation or Cell Counting Kit-8 (CCK-8) assays. For the colony formation assay (19, 20), cells were seeded into 6-well plates at a density of 1,000 cells/well. Twenty-four hours later, the cells were treated with X-rays or drugs at the indicated doses and then cultured for 10 to 14 days, followed by staining with 0.5% crystal violet. Colonies containing more than 50 cells were counted. For the CCK-8 assay (21), cells (3,000/well) were cultured in 96-well plates for 24 hours and then treated with drugs for 72 hours. Cell viability was determined by OD_{540 \text{ nm}} using CCK-8 (Dojindo). The IC_{50} was calculated using GraphPad Prism 5.0 (GraphPad Software).

Apoptosis assay
Apoptosis was evaluated with an Annexin V/propidium iodide (PI) double-staining assay, as described previously (22, 23). Briefly, cells were treated with X-rays, cultured for 48 hours,
RFC4, a Radioresistance Factor, Promotes NHEJ Repair in Colorectal Cancer

RFC4 is identified as a candidate radioresistance factor facilitating colorectal cancer cell survival in response to X-ray exposure in vitro. A, The schematic procedure of genome-wide RNAi screening that identified 6 candidate radioresistance genes. B–D, Colony formation assays were used to assess survival at 10 days after exposure to X-rays in HCT116 cells with stable gene knockdown (shNC, negative control shRNA; shGENE, GENE-specific shRNA; B), colorectal cancer cells with RFC4 knockdown or overexpression (top, WB; Vec, control lentiviral vector; RFC4, RFC4 overexpression lentiviral vector; sh1/2, RFC4 knockdown by shRFC4-1/2; C), and colorectal cancer cells with endogenous RFC4 silencing by stable transfection of sh5 (a shRNA targeting the RFC4 3'-UTR) after restoring RFC4 expression by transient transfection of the RFC4 expression plasmid (left, representative colony formation pictures and WBs; right, bar charts indicating the mean number of colonies/well; Vec, pcDNA4.0; RFC4, RFC4 cDNA in pcDNA4.0; D). E and F, Assessment of the effects of RFC4 on apoptosis 48 hours after cells were treated with X-rays by Western blot (loading control: actin; E) and Annexin V/PI double-staining assays with flow cytometry (the percentage of apoptotic cells is presented as the mean ± SD of at least three independent experiments; F; *, P < 0.05; **, P < 0.01; ***, P < 0.001).
harvested, and stained using an Annexin V/PI Apoptosis Kit (BestBio). The samples were then analyzed by flow cytometry.

Coimmunoprecipitation and Western blot analyses

Coimmunoprecipitation (Co-IP) and Western blot (WB) analyses were performed as described previously (14). Briefly, cells were lysed using lysis buffer (Cell Signaling Technology) supplemented with protease (Roche) and phosphatase (KeyGen Biotech) inhibitors, and the protein concentration was quantified using a BCA Protein Assay Kit (KeyGen Biotech). For Co-IP, cell lysates were incubated with M2 anti-IgG agarose (A2220; Sigma-Aldrich) and anti-Ku80 (111; Invitrogen) antibodies plus protein G agarose beads (Santa Cruz Biotechnology). After washing, the pull-down products were examined by mass spectrometry or Western blot analysis.

For Western blot analysis, samples were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Sigma-Aldrich), which was blocked with 5% milk in Tris-buffered saline and incubated overnight at 4°C with specific antibodies against the following proteins: RFC4 (PA5-21538) and Ku80 (111; Invitrogen); and GAPDH (D16H11), β-actin (8H10D10), Ku70 (D010A7), cleaved PARP-1 (D64E10), cleaved caspase-3 (#9661), γH2AX (D17A3), p-ATM (Ser1981) (D25E5), and proliferating cell nuclear antigen (PCNA; D3H8P; Cell Signaling Technology). Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (#7074) or anti-mouse IgG (#7076P2; Cell Signaling Technology). Signals were visualized using an ECL detection system (Amersham Biosciences).

Immunofluorescence

Cells in confocal dishes were fixed in 4% paraformaldehyde, permeabilized using 0.5% Triton X-100, and blocked with 3% BSA (Beyotime). The cells were then incubated with anti-γH2AX (1:50; D17A3; Cell Signaling Technology) or anti-RAD51 (1:50; 3C10; Thermo Fisher Scientific) antibodies at 4°C overnight, followed by incubation with goat anti-rabbit Alexa Fluor 594 (#A1012) or goat anti-mouse Alexa Fluor 488 (#A11001; 1:200; Invitrogen) for 1 hour at room temperature in the dark. The samples were costained with DAPI (Beyotime) and examined by confocal laser scanning microscopy (20).

Comet assay

DNA damage was assessed using the comet assay (24). Cells were treated with X-rays (6 Gy), cultured for 6 hours, and then stained using an Annexin V/PI Apoptosis Kit (KeyGen Biotech). The samples were then analyzed by flow cytometry.

NHEJ and homologous recombination reporter assays

Reporter constructs for NHEJ and homologous recombination (HR; refs. 25, 26) and the I-SceI expression plasmid pCMV-NLS-I-SceI were gifts from Dr. Lin Feng, Sun Yat-sen University Cancer Center (SYSUCC, Guangzhou, China). The structure and function of the NHEJ and HR reporters are presented in Supplementary Fig. S2. For NHEJ and HR reporter assays (25), HCT116 and HT29 cells were stably transfected with NHEJ or HR reporter constructs and selected using G418 (1 mg/mL; Beyotime) for 10 days. G418-resistant cells were utilized as models to analyze chromosomal DSB repair. These cells were cotransfected with pCMV-NLS-I-SceI and the indicated shRNAs or siRNAs. After 48 hours of incubation, the cells were harvested to assess the proportion of GFP+ cells using a CytoFLEX flow cytometer (Beckman). The NHEJ- or HR-mediated DNA repair efficiency (NHEJ or HR activity) is presented as the percentage of GFP+ cells normalized to the respective control.

Random plasmid integration assay

NHEJ-mediated repair was assessed using a cell-based plasmid integration assay, as previously described by Feng and Chen (26), with minor modifications. Briefly, cells were transiently cotransfected with an EcoRV/BamHI-linearized pcDNA3.1/zeo(+) plasmid (Invitrogen) and the pEGFP-C1 plasmid (Clontech). Twenty-four hours later, the cells were collected, counted, and plated into two dishes. One day after plating, the cells in one dish were used to determine the transfection efficiency by assessing EGFP expression (green fluorescence), and the cells in the other dish were incubated in selective media containing 300 μg/mL zeocin (Syngene) for 14 days at 37°C to allow colony formation. Colonies indicating random plasmid integration were counted after crystal violet staining. Colony number was normalized to the respective transfection efficiency, and NHEJ activity is shown as a percentage relative to the control group.

Nude mouse xenograft model and radiotherapy

Animal experiments were approved by the SYSUCC Institutional Animal Care and Usage Committee following the Animal Welfare and Rights in China (Approval No.: L102012018006K). Female BALB/c nude mice (4–5 weeks old; 15–18 g; SLRC Laboratory Animal Co.) were used to generate xenograft models via subcutaneous transplant of tumor sections (approximately 5 mm3) from colorectal cancer cell xenografts into the right flank. Once the xenograft volume reached approximately 150 mm3, the mice were treated with X-rays at 2 Gy/day for 7 consecutive days. A lead plate was used to cover the mouse body except the xenograft region. Xenograft growth was monitored, and tumor volume was calculated using the following formula: tumor volume = 0.52 × width2 × length (14, 22). The mice were sacrificed 4 weeks after radiotherapy, and the tumors were removed, weighed, and subjected to pathologic analysis.

Patients

A total of 145 patients with LARC with newly diagnosed LARC who received neoCRT from May 2005 to May 2016 at SYSUCC were included in this study. For inclusion, the patients were required to have a single primary lesion, have completed standard neoCRT and undergone radical surgical resection, and have survived more than 1 month after surgery; complete information was also an inclusion criterion (27). The study was approved by the SYSUCC Ethics Committee (Approval No.: GZR2018-164) and conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from each patient. Pre- and post-neoCRT tumor tissues were obtained by colonoscopy or during surgery, respectively, and then paraffin-embedded for IHC. All patients received standard neoCRT with a total dose of 50 Gy (2 Gy/day, 5 days/week, 5 weeks) plus concurrent chemotherapy with fluorouracil (capecitabine 825 mg/m2 days 1–5, repeated every week; or 5-FU 400 mg/m2 and leucovorin 400 mg/m2 day 1 and 5-FU 1,200 mg/m2 continuous intravenous
infusion (c.i.v.) 48 hours, repeated every 14 days) or fluorouracil+oxaliplatin regimen [XELOX (oxaliplatin 100 mg/m² day 1, capecitabine 1,000 mg/m² twice a day for days 1–14, repeated every 21 days) or mOLFFOX6 (oxaliplatin 85 mg/m², leucovorin 400 mg/m², and 5-FU 400 mg/m², day 1; 5-FU 1,200 mg/m² c.i.v. 48 hours; repeated every 14 days)].

Tumor responsiveness to neoCRT was evaluated using the tumor regression grade (TRG), as proposed in the 6th Edition of the American Joint Committee on Cancer (AJCC) Staging Manual System, which is based on residual neoplastic cells, cytologic changes (including nuclear pyknosis or necrosis and/or eosinophilia), and stromal changes (including dense or edematous fibrosis with or without inflammatory infiltrate and giant cell granulomatous around ghost cells and keratin; ref. 28).

After surgery, 124 of the patients received and completed adjuvant chemotherapy with a capecitabine, XELOX, or mOLFFOX6 regimen. The detailed characteristics of the patients are presented in Supplementary Table S4.

IHC assay

IHC assays were conducted to detect protein levels in tissues, as described previously (29). Briefly, 4-μm-thick formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated, followed by antigen retrieval and endogenous peroxidase inactivation. After blocking, the slides were incubated overnight at 4°C with anti-RFC4 (1:400; #PA5-21538; Thermo Fisher Scientific), anti-cleaved caspase-3 (1:200, #9661; Cell Signaling Technology), or anti-yH2AX (1:100; D17A3; Cell Signaling Technology) antibodies; incubated with a HRP-conjugated secondary antibody; visualized using an Envision Detection Kit (Dako); and counterstained with hematoxylin. RFC4 levels were independently and semiquantitatively assessed by two pathologists using the immunoreactive score (IRS), which was calculated as the product of the intensity score (0, absent; 1, weak; 2, moderate; and 3, strong; Supplementary Fig. S3) and the positive cell percentage score (0, 0%; 1, <10%; 2, 11%–50%; 3, 51% to 80%; and 4, ≥80%); thus, the IRS ranged from 0 to 12 (30). The cut-off value for RFC4 level was deduced according to overall survival (OS) using an ROC curve, and patients were categorized into two groups based on RFC4 level (high or low; ref. 19).

Statistical analysis

All in vitro experiments were repeated at least three times, and the animal experiments were repeated twice. The data were analyzed using GraphPad Prism 5.0 or SPSS 20.0 software (SPSS Inc.). Differences in the average among two or more groups were compared using Student t test or ANOVA; associations between RFC4 levels and clinicopathologic parameters were assessed using the $\chi^2$ test. Survival was analyzed using the Kaplan–Meier method and the log-rank test; associations of variables with survival were analyzed using a univariate or multivariate Cox proportional hazards model. A P value <0.05 was considered to indicate a significant difference.

Results

Identification of RFC4 as a radioresistance factor in colorectal cancer

To identify radioresistance factors in colorectal cancer, we developed a high-throughput RNA interference screen using the LentiPlex human whole-genome shRNA library. Two colorectal cancer cell lines with different genetic backgrounds, HCT116 and HT29 cells, were infected with the lentiviral shRNA library and then exposed to X-rays after puromycin selection. shRNA sequences and copy numbers were examined in untreated cells and in those that survived 6 Gy of X-ray exposure; following MAGeCK-negative selection, 757 and 565 candidate genes with a depleted shRNA copy number in surviving cells (Supplementary Tables S5 and S6) were selected in HCT116 and HT29 cells, respectively. The intersection of these two candidate gene sets identified 6 genes, RFC4, NCAPH, SYNE3, LDLRAD2, NHP2, and FICD, as candidate radioresistance genes (Fig. 1A). The copy number changes of shRNAs targeting these 6 genes in HCT116/HT29 cells are shown in Supplementary Fig. S4A, and these shRNA sequences are listed in Supplementary Table S2.

To confirm the screening results, we constructed HCT116 cell lines with stable gene knockdown (Supplementary Fig. S4B) via infection with lentiviruses encoding representative shRNAs (Supplementary Table S2) targeting these 6 candidate genes; we then performed a clonogenic assay after exposure to X-rays. The results showed that knockdown of RFC4, NCAPH, and FICD significantly impaired radioresistance in HCT116 cells; indeed, cells with RFC4 knockdown were the most sensitive to X-rays (Fig. 1B; Supplementary Fig. S4C). In addition, comparative expression analysis of these 6 genes revealed significantly increased mRNA levels of RFC4, NCAPH, and FICD in radio-resistant HCT116+GyR cells compared with parental HCT116 cells, and the increase in RFC4 mRNA was the most notable (Supplementary Fig. S4D).

Furthermore, radiation induced the upregulation of RFC4 expression in HCT116 cells in vitro in a dose-dependent manner, and RFC4 protein levels were significantly higher in residual tumor tissue after neoCRT than in biopsy specimens before neoCRT (Supplementary Fig. S5A–S5C). These findings indicate that RFC4 may be an important factor in the radiosensitivity of colorectal cancer.

RFC4 facilitates colorectal cancer cell survival after X-ray exposure in vitro

To further explore the effect of RFC4 on radiosensitivity in colorectal cancer, we stably overexpressed RFC4 in HCT116 and HCT15 cells with lower RFC4 levels, knocked down RFC4 in HT29 and SW480 cells with higher RFC4 levels (Supplementary Fig. S5D), and performed clonogenic assays after X-ray treatment. The results showed that RFC4 overexpression significantly enhanced colony formation in HCT116 and HCT15 cells after radiation, whereas RFC4 knockdown significantly weakened colony formation in HT29 and SW480 cells after radiation (Fig. 1C; Supplementary Fig. S6). Moreover, restoring RFC4 expression in RFC4-silenced colorectal cancer cells via transient transfection of an RFC4 expression plasmid rescued the impaired colony formation after radiation (Fig. 1D).

The Western blot results showed that PARP-1 and caspase-3 cleavage induced by radiation was inhibited in RFC4-overexpressing HCT116 cells and enhanced in RFC4-silenced HT29 cells compared with the respective controls (Fig. 1E). Annexin V/PI double-staining assays revealed that RFC4 overexpression suppressed radiation-induced apoptosis in HCT116 cells and that RFC4 knockdown promoted radiation-induced apoptosis in HT29 cells (Fig. 1F; Supplementary Fig. S7). These findings indicate that RFC4 protects colorectal cancer cells from radiation-induced apoptosis.
RFC4 promotes colorectal cancer cell radioresistance in a nude mouse xenograft model. A and B, Assessment of radiotherapy efficacy in colorectal cancer xenografts in nude mice. Transplanted xenografts were established with RFC4-overexpressing (RFC4) and vector-transfected (Vec) HCT116 cells (A) or RFC4-knockdown (sh1/sh2) and negative control (shNC) HT29 cells in the right flank of BALB/c nude mice, which were treated with X-rays (2 Gy/day) for 7 consecutive days; tumor volume and weight were measured (left, removed xenografts; middle, tumor volume; right, average tumor weight; B). C, Representative images of IHC staining for RFC4 (top) and cleaved caspase-3 (middle) in the harvested xenografts and H&E staining (bottom) showing the distribution of tumor cells (red arrow) and the infiltration of inflammatory cells (green arrow; **, P < 0.01; ***, P < 0.001; n.s., not significant).

RFC4 promotes the radioresistance of colorectal cancer cells in a nude mouse xenograft model

Next, we investigated the effect of RFC4 on the response to radiation in a nude mouse xenograft model. After establishment of subcutaneous colorectal cancer xenografts, the mice were treated with X-rays (2 Gy/day for 7 consecutive days). As shown in Fig. 2A, the xenografts originating from HCT116 cells overexpressing RFC4 (HCT116-RFC4) were larger and heavier
than those originating from vector-transfected HCT116 cells (HCT116-vec) after radiotherapy ($P < 0.001$). Furthermore, the tumor inhibition ratio (radiotherapy effect) was significantly attenuated in the HCT116-RFC4 group compared with the HCT116-vec group (45.4% vs. 86.6%). Conversely, the xenografts derived from HT29 cells with RFC4 knockdown (HT29-sh1/sh2) were smaller and weighed less than those derived from HT29-shNC cells after radiotherapy ($P < 0.01$), and the radiotherapy effect was markedly enhanced in the HT29-sh1/sh2 groups compared with the HT29-shNC group (92.4% and 95.1% vs. 75.9%; Fig. 2B). IHC and hematoxylin and eosin (H&E) staining assays showed weaker cleaved caspase-3 staining and less inflammatory cell infiltration in RFC4-overexpressing HCT116 xenografts, and the opposite effects were found in RFC4-silenced HT29 xenografts (Fig. 2C). These findings indicate that RFC4 confers radioresistance on colorectal cancer cells by protecting them from radiation-induced apoptosis both in vitro and in vivo.

RFC4 promotes radioresistance by facilitating DSB repair in colorectal cancer cells

It is well known that radiation causes cellular DNA DSBs and that phospho–H2AX (γ-H2AX) levels and foci are sensitive DSB markers. Therefore, we investigated the influence of RFC4 on γ-H2AX levels in X-ray–treated colorectal cancer cells by Western blot and IF assays. The results showed lower γ-H2AX levels and fewer foci in HCT116-RFC4 cells than in HCT116-vec control cells at 12 hours posttreatment with 6 Gy X-rays; conversely, significantly higher γ-H2AX levels and more foci were observed in HT29-sh1/sh2 cells than in HT29-shNC cells at 12 hours after irradiation (Fig. 3A and B). Moreover, IHC assays indicated significantly lower γ-H2AX levels in HCT116-RFC4 xenografts than in HCT116-vec xenografts at 4 weeks after radiotherapy but higher γ-H2AX levels in HT29-sh1/sh2 xenografts than in control xenografts (Fig. 3C).

Next, we measured DSB levels by the comet assay, in which the mean tail moment after X-ray treatment was quantified using image analysis. We found that HCT116-RFC4 cells had significantly shorter comet tails and that HT29-sh1/sh2 cells displayed longer comet tails than did their control cells at 6 hours after radiation exposure (Fig. 3D).

To clarify whether RFC4 affects radiation-induced DNA damage or DNA repair, we detected γ-H2AX levels at different times after radiation. The results demonstrated that γ-H2AX levels immediately and dramatically increased after radiation, peaking at 0.5 hour in HCT116 cells and at 2 hours in HT29 cells, and then gradually decreased over time. No obvious difference in γ-H2AX levels was found between HCT116-RFC4/HT29-sh1 cells and their relevant control cells before γ-H2AX levels peaked; however, γ-H2AX levels decreased more rapidly in colorectal cancer cells with higher RFC4 levels (HCT116-RFC4 or HT29-shNC) than in those with lower RFC4 levels (HCT116-vec or HT29-sh1, respectively; Fig. 3E; Supplementary Fig. S8A). Further investigation revealed that ATM phosphorylation levels were similar in colorectal cancer cells with high or low RFC4 levels in the early stages after X-ray exposure (Supplementary Fig. S8B). These results suggest that RFC4 may not affect early activation of the DNA damage response but might promote consequent DNA repair. Taken together, the data indicate that RFC4 promotes DNA DSB repair in colorectal cancer cells.

RFC4 is involved in NHEJ-mediated DNA repair

As NHEJ and HR are the two main DSB repair pathways, we first determined which pathway is influenced by RFC4 in DSB repair in colorectal cancer cells using NHEJ and HR reporter systems (Supplementary Fig. S2; ref. 25). The results showed that RFC4 knockdown significantly decreased NHEJ activity (Fig. 4A) but did not affect HR activity (Fig. 4B), suggesting that RFC4 is likely involved in NHEJ but not in HR during DSB repair in colorectal cancer cells.

Because the random integration of plasmids into genomic DNA is considered an NHEJ-mediated DNA repair mechanism (26, 31), we performed a random plasmid integration assay to confirm whether RFC4 can regulate NHEJ repair. Consistent with the NHEJ reporter assay results, RFC4-overexpressing cells exhibited a significantly enhanced NHEJ repair capacity, whereas RFC4-silenced cells displayed reduced NHEJ activity (Fig. 4C; Supplementary Fig. S9A). In addition, we detected RAD51 foci, a core sign of HR repair (32), and found no difference in the number of RAD51 foci between RFC4-knockdown colorectal cancer cells and control cells (Supplementary Fig. S9B). These data suggest that RFC4 facilitates NHEJ-mediated DSB repair in colorectal cancer cells.

We also investigated the effects of other components of the RFC complex on the NHEJ pathway. Similar to RFC4, knockdown of RFC5 significantly decreased NHEJ activity, but knocking down RFC1, RFC2, or RFC3 had no obvious effect on NHEJ pathway activity (Supplementary Fig. S9C).

RFC4 regulates NHEJ repair by interacting with Ku70/80

To investigate the molecular mechanism by which RFC4 regulates NHEJ repair in colorectal cancer cells, we screened proteins interacting with RFC4 via IP and mass spectrometry (Supplementary Tables S7 and S8) and found that both Ku70 and Ku80, which are core components of the NHEJ complex, bind to RFC4 in HCT116 and HT29 cells. Furthermore, co-IP–Western blot assays revealed that Ku70 and Ku80 were pulled down by RFC4 (FLAG) in lysates of both radiation-treated and untreated HCT116 cells (Fig. 4D). Moreover, endogenous RFC4 was pulled down by both Ku70 and Ku80 in lysates of HCT116 cells treated with or without X-rays (Fig. 4E). To verify the impact of RFC4 binding to Ku70 and Ku80, we examined the effects of RFC4 on radiosensitivity after silencing Ku70 and Ku80. The results showed that Ku70 or Ku80 silencing almost completely abrogated the RFC4-induced radiosensitivity of HCT116 cells (Fig. 4F), suggesting that the ability of RFC4 to promote radioresistance in colorectal cancer may depend on its interaction with Ku70/80.

As it has been reported that the RFC complex interacts with PCNA and promotes its loading onto chromatin, which is involved in DNA damage repair (33, 34), we investigated whether PCNA is involved in regulating RFC4 during DSB repair in colorectal cancer cells. Although RFC4 overexpression increased radiosensitivity similarly in PCNA-silenced HCT116 cells and control cells, PCNA silencing significantly impaired radiation resistance (Supplementary Fig. S9D). Taken together, our data indicate that RFC4 interacts with Ku70 and Ku80 to promote the NHEJ pathway, which is involved in DSB repair in colorectal cancer cells.

High RFC4 levels are associated with radioresistance and poor prognosis in patients with LARC treated with neoRT

Because RFC4 was identified as a colorectal cancer radioresistance factor in our cellular and animal experiments, we sought to...
RFC4 promotes radioresistance by facilitating DNA DSB repair in colorectal cancer cells. A and B, The effects of RFC4 on γ-H2AX levels and foci in colorectal cancer cells treated with X-rays by Western blot (A) and IF (B: left, representative IF images; right, foci number is presented as the mean ± SD of three independent experiments). C, IHC assay for γ-H2AX levels in harvested xenografts (representative images). D, Comet assay for DNA damage in colorectal cancer cells at 6 hours after treatment with 6 Gy X-rays (left, representative pictures; right, bar charts indicating the average tail moment per cell). E, Western blot analysis of the time course of changes in γ-H2AX levels in colorectal cancer cells treated with X-rays (loading control: GAPDH). Vec, lentiviral vector; RFC4, lentiviral vector expressing RFC4; shNC, negative control shRNA; sh1/2, shRNA shRFC4-1/2; **, P < 0.01.)
RFC4, a Radioresistance Factor, Promotes NHEJ Repair in Colorectal Cancer

Figure 4. RFC4 promotes NHEJ-mediated DSB repair by interacting with Ku70/Ku80. NHEJ (A) and HR reporter assays (B) showing the effects of RFC4 on DNA DSB repair in HT29 cells (top, NHEJ or HR repair activity presented as the relative GFP+ fraction normalized to the shNC group; bottom, Western blot analysis of RFC4 expression). C, Relative NHEJ activity in colorectal cancer cells was assessed using a random plasmid integration assay. D and E, Co-IP and Western blot analyses. HCT116-Vec (−) and HCT116-RFC4 (+) (D) or HCT116 cells were treated with X-rays (0 or 6 Gy; E). After 12 hours, cell lysates were immunoprecipitated with Flag (D) or Ku70/Ku80 antibodies (E), followed by Western blot using the indicated antibodies.

Using ROC curve analysis, we obtained a cut-off value for RFC4 levels in colorectal cancer tissues, 6.8; this value optimally classified patients into two groups with high or low RFC4 levels. Further analysis showed that a high RFC4 level was significantly associated with a high TRG ($P < 0.001$) and poor survival ($P = 0.034$; Supplementary Table S4). Kaplan–Meier survival analysis indicated that patients with high RFC4 levels had a shorter progression-free survival (PFS, $P = 0.035$) and OS ($P = 0.006$; Fig. 5B and C). Moreover, multivariate Cox proportional

determine whether RFC4 has consistent effects on radiotherapy in clinical patients with colorectal cancer. Thus, we investigated RFC4 expression levels in pretreatment biopsy tumor specimens from 145 patients with LARC who received neoCRT followed by surgery with or without adjuvant chemotherapy and analyzed the relationship between RFC4 levels and TRG. According to our results, RFC4 levels were significantly lower in tumor tissues from patients with TRG 0 (pathologic complete response) than in those from patients with TRG 1–3 (Fig. 5A).
hazard regression analysis revealed that the RFC4 level, together with the pathologic grade and/or surgical procedure, could serve as an independent prognostic factor (Table 1). These findings indicate that high RFC4 levels in colorectal cancer tissues may result in resistance to neoCRT.

Regardless, we could not distinguish whether RFC4 confers resistance to radiotherapy, chemotherapy, or both based on these data. Therefore, we performed in vitro cell viability and colony formation assays using colorectal cancer cells to investigate the effects of RFC4 on resistance to the chemotherapy drugs 5-fluorouracil, oxaliplatin, doxorubicin, and etoposide. Overall, RFC4 promoted colorectal cancer cell resistance to doxorubicin and etoposide, which usually cause cellular DNA damage, but not to 5-fluorouracil and oxaliplatin, which were included in the chemotherapy regimens for the abovementioned patients (Supplementary Fig. S10). These findings suggest that high RFC4 expression confers resistance to radiotherapy but not to chemotherapy in neoCRT. Altogether, RFC4 was found to potentially confer radioresistance in colorectal cancer; thus, a high RFC4 level might predict poor tumor regression and prognosis in patients with LARC receiving preoperative neoRT.

**Discussion**

RFC is a conserved eukaryotic complex consisting of five distinct subunits, a large subunit, RFC1, and four small subunits, RFC2 to RFC5, which play essential roles in DNA replication and DNA damage checkpoints in Saccharomyces cerevisiae (38).

**Table 1.** Univariate and multivariate Cox regression analyses of prognostic factors for patients with LARC

<table>
<thead>
<tr>
<th>Variables</th>
<th>PFS</th>
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<th>OS</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
<td>Univariate</td>
<td>Multivariate</td>
</tr>
<tr>
<td>Gender (male vs. female)</td>
<td>1.947 (1.035–3.662)</td>
<td>0.035&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.341 (0.659–2.727)</td>
<td>0.417</td>
</tr>
<tr>
<td>Age (&gt;57 years vs. ≥57 years)</td>
<td>0.993 (0.553–1.848)</td>
<td>0.982</td>
<td>1.905 (0.595–2.400)</td>
<td>0.616</td>
</tr>
<tr>
<td>Pathologic grade (III vs. II)</td>
<td>3.203 (1.698–6.042)</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.022 (1.491–6.125)</td>
<td>0.001&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>cT (T4 vs. T2–T3)</td>
<td>1.138 (0.611–2.122)</td>
<td>0.683</td>
<td>1.252 (0.625–2.508)</td>
<td>0.526</td>
</tr>
<tr>
<td>cN (N1–N2 vs. N0)</td>
<td>0.675 (0.356–1.282)</td>
<td>0.227</td>
<td>0.818 (0.394–1.700)</td>
<td>0.590</td>
</tr>
<tr>
<td>CEA (ng/mL) (&gt;5 vs. &lt;5)</td>
<td>1.599 (0.852–3.001)</td>
<td>0.141</td>
<td>1.718 (0.847–3.483)</td>
<td>0.129</td>
</tr>
<tr>
<td>CA199 (kU/L) (&gt;35 vs. ≤35)</td>
<td>2.219 (1.092–4.508)</td>
<td>0.024&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.822 (0.806–4.119)</td>
<td>0.144</td>
</tr>
<tr>
<td>Neoadjuvant chemotherapy (FU vs. FU+Oxa)</td>
<td>0.657 (0.248–1.737)</td>
<td>0.393</td>
<td>0.727 (0.215–2.478)</td>
<td>0.609</td>
</tr>
<tr>
<td>Adjvant chemotherapy (yes vs. no)</td>
<td>0.429 (0.209–0.881)</td>
<td>0.018&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.389 (0.178–0.852)</td>
<td>0.015&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Surgical procedure (Dixon/Hartman vs. Miles)</td>
<td>2.166 (1.155–4.060)</td>
<td>0.014&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.378 (1.173–4.819)</td>
<td>0.013&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>TRG (2–3 vs. 0–1)</td>
<td>2.141 (1.349–3.991)</td>
<td>0.014&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.129 (1.058–4.282)</td>
<td>0.030&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>RFC4 level (high vs. low)</td>
<td>2.133 (1.038–4.381)</td>
<td>0.035&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.266 (1.079–4.759)</td>
<td>0.031&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
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</table>

Abbreviations: CI, confidence interval; cN, clinical N stage; cT, clinical T stage; FU, capecitabine or 5-fluorouracil; FU+Oxa, FU+leucovorin; XELOX, or mFOLFOX6.

<sup>a</sup>P < 0.05.

<sup>b</sup>P < 0.001.

<sup>c</sup>P < 0.001.
It is well known that radiation induces cellular DNA DSBs to kill tumor cells and that intensive repair of DSBs results in radioresistance in cancer cells (39, 40). HR and NHEJ are the major DSB repair pathways in higher eukaryotes (41, 42). HR results in precise repair of the DNA lesion but requires the presence of homologous sequences, whereas NHEJ results in error-prone repair because of its independence of DNA sequence homology. In this study, RFC4 was identified as a radioresistance factor that facilitates NHEJ but not HR in DSB repair via interactions with Ku70/Ku80 (Fig. 4). The core NHEJ complex consists of Ku80 (XRCC5), Ku70 (XRCC6), and DNA-dependent protein kinase, and formation of the Ku70-Ku80 heterodimer and subsequent binding to DNA DSB ends are the initiating events of NHEJ (43, 44). Therefore, we speculate that RFC4 may promote Ku80–Ku70 heterodimer recruitment and retention at DSB sites in colorectal cancer cells. Knockdown of Ku70 or Ku80 almost completely reversed RFC4-induced radiation resistance in colorectal cancer cells (Fig. 4F), suggesting that the interaction of RFC4 with Ku70/Ku80 is required for efficient NHEJ repair.

In general, RFC4 functions as a component of the RFC complex (45). However, we found that knocking down RFC4 or RFC5, but not other RFC subunits, significantly reduced NHEJ activity (Supplementary Fig. S9C). Moreover, RFC4 was the only RFC component selected in our high-throughput RNA interference screen (Supplementary Tables S3 and S6). Thus, it is essential to further investigate whether RFC4 regulates NHEJ-mediated DNA repair by itself or as part of the complete RFC complex.

It has been reported that RFC is involved in nucleotide excision repair by loading the PCNA sliding clamp complex onto DNA (36). However, our study showed that PCNA knockdown did not impair RFC4-enhanced radioresistance in colorectal cancer cells (Supplementary Fig. S9D), suggesting that the effects of RFC4 on the repair of radiation-induced DNA damage may be independent of PCNA loading onto DNA. Our findings may provide a novel regulatory mechanism for NHEJ-mediated DSB repair.

Resistance to CRT is the main cause of treatment failure or relapse in colorectal cancer (45, 46). Our clinical investigation revealed that higher RFC4 expression in cancer cells was significantly associated with weaker tumor regression after neoCRT (Supplementary Table S4); moreover, RFC4 upregulation predicted a poor prognosis in patients with LARC who received neoCRT (Fig. 5). Indeed, patients with LARC with lower RFC4 expression were more likely to experience better tumor regression and survival outcomes. In addition to the long-course radiotherapy used in this study, short-course radiotherapy (5 Gy/day, 5 days/week, 1 week) is administered as neoadjuvant therapy in some patients with stage T3 rectal cancer, based on NCCN guidelines. According to data obtained from a nude mouse xenograft experiment, RFC4 promoted resistance to X-rays in colorectal cancer cells after only 7 consecutive days of treatment. Thus, we presume that RFC4 might also be involved in resistance to short-course radiotherapy, which should be examined in future studies. We also found that RFC4 overexpression greatly enhanced the resistance of colorectal cancer cells to doxorubicin and etoposide but did not affect their sensitivity to 5-fluorouracil or oxaliplatin. Fluorouracil is an anticancer antimetabolite, and platinum causes major single-strand DNA damage (47); doxorubicin and etoposide mainly cause DSBs in tumor cells (48). Therefore, we speculate that RFC4 might promote the repair of DSBs induced by radiation or chemotherapy and then increase the resistance of colorectal cancer cells to these treatments with no obvious effect on other treatments. Considering that 5-fluorouracil and oxaliplatin, but not doxorubicin and etoposide, are commonly used in colorectal cancer chemotherapy, the impaired tumor regression and poor prognosis of patients with LARC treated with neoCRT are likely due to radiation resistance resulting from strong RFC4 expression in cancer cells, not resistance to fluorouracil and/or oxaliplatin.

RFC4 was found to be highly expressed in cancer tissues and cells, including colorectal cancer (49), and RFC4 expression is associated with tumor progression and prognosis in patients with colorectal cancer (50). Nonetheless, according to the results of our investigation and a previous report (50), RFC4 levels were not correlated with the T and N stages of patients with colorectal cancer. Moreover, RFC4 did not affect the colony formation ability of colorectal cancer cells in vitro (Supplementary Figs. S6 and S10B) or the growth of colorectal cancer xenografts in nude mice (Fig. 2), even though RFC4 plays an important role in radioresistance. Therefore, the influence of RFC4 on colorectal cancer tumorigenesis and progression is still unknown. In addition, an analysis of a cohort of patients with early-stage colorectal cancer who received only surgery but not neoadjuvant or adjuvant therapy is needed to clarify the relationship between RFC4 expression and colorectal cancer progression and prognosis.

Frequent amplification of the RFC4 gene has been reported in lung, breast, and cervical cancer, but RFC4 gene amplification has not been detected in colorectal cancer (49). We observed that radiation induced the upregulation of RFC4 expression in colorectal cancer tissues and cells and that RFC4 levels were higher in radioresistant colorectal cancer cells than in parental cells (Supplementary Figs. S4D and S5). We presume that the radiation-induced upregulation of RFC4 is likely one of the causes of induced secondary radioresistance. However, the exact causes and mechanisms of RFC4 upregulation in colorectal cancer tissues need to be further clarified in future investigations.

In summary, RFC4 was found to act as a resistance factor that protects colorectal cancer cells from radiation-induced DSBs and apoptosis in vitro as well as in nude mouse colorectal cancer xenografts. Mechanistically, RFC4 promoted NHEJ-mediated DNA DSB repair by interacting with Ku70/Ku80. Moreover, RFC4 upregulation in cancer cells was associated with reduced tumor regression and poor prognosis in patients with LARC treated with neoRT. Therefore, RFC4 levels might serve as an effective predictive biomarker of radiation sensitivity and a target for radiosensitization in patients with LARC; these findings may contribute to the development of precise treatment strategies in clinical practice in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: X. Yue, W. Huang, R.-Y. Liu
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X.-C. Wang, X. Yue, L.-Y. Le
Writing, review, and/or revision of the manuscript: X.-C. Wang, X. Yue, R.-Y. Liu

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Study supervision: Z.-Z. Pan, W. Huang, R.-Y. Liu

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